EFFECTS OF COLLAGEN CROSSLINKING ON MECHANICAL BEHAVIOR OF CORNEAL TISSUE IN COMPRESSION

By

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EFFECTS OF COLLAGEN CROSSLINKING ON MECHANICAL BEHAVIOR OF CORNEAL TISSUE IN COMPRESSION

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Abstract:

Eyesight is crucial to many daily tasks. Proper eyesight depends on soft tissues that seem simple at the macroscale, but have complicated microstructures necessary for proper function. The cornea is the first thing that light encounters on its path into the eye. Within the cornea are multiple distinct layers with each accomplishing a different function. This tissue must be strong as it helps support the shape of the eye. It also must remain flexible as it needs to cope with pressure changes due to biology as well as accidental impacts. Keratoconus is an eye disorder in which the cornea becomes thin; therefore, the cornea loses its ability to withstand the intraocular pressure and distorts causing increasingly fuzzy vision. So far there have been different procedures for treating this disease state but none of them have been successful in preventing the progression of the disease. Nevertheless, corneal crosslinking by using riboflavin (a noninvasive procedure) has the promise of halting this deformation process, but is still not fully understood. The primary objective of this thesis is to investigate the effect of collagen crosslinking on the mechanical behavior of the cornea in compression. The differences in compressive behaviors between three species are also studied. Results suggest that corneal collagen crosslinking has only a minimal effect on the compressive properties. Results also show that a porcine model more closely mimics that of human corneal tissues than does a bovine model.

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CHAPTER I

INTRODUCTION

1.1 Motivation

Eyesight is crucially important throughout people's lives. The first thing we do in the morning is to look at an alarm clock or the sun shining through the windows. Whether it is walking through a crowded area, doing exercise, enjoying hobbies, or even every day chores like cooking a meal, eye sight is almost always needed. Sight is a complex ability that relies on both external environment and an individual's physiology. First light must come from the environment. The brightness must be neither too bright as to hurt our eyes nor too dim for us to pick up. Furthermore, and the wavelength must be within the small range suitable for human capabilities. After light reaches our eyes it must pass through the solid yet clear cornea, through a fluid called the aqueous humor, then to the lens. The lens must focus the stream of light through the vitreous humor (a fluid which supports the lens) onto the back of the eye. This must occur every moment in a nearly perfect manner if the nerves have any chance at taking the information to the brain.

The first physiological component of sight is the cornea. Ideally this structure is a tough, elastic and clear solid which protects the rest of the inner eye from physical damage. The cornea must allow light to pass easily through and still be able to provide structural support by means of withstanding the intraocular pressure that inflates the eye. With so many roles for such a small tissue there are also many things that can go wrong. Problems can arise from bacterial infections or physical abrasion which are both painful and may leave a scar which impedes light. The cornea may also experience abnormal blood vessel growth (vascularization), inflammations (via ulcers or keratitis) and physically based issues such as excess hydration (Fuch's dystrophy) or excessive deformation due to stretching (keratoconus). Multiple genetic disorders can also cause a buildup on the eye and are grouped together as dystrophies.

During keratoconus, for reasons yet unknown the central corneal tissue thins. This thinning causes the tissue to stretch under the intraocular pressure and has been of interest to both the medical and scientific communities. The deformation of the corneal shape diverts light entering the eye from its normal path causing blurred vision. This incurable condition is typically is first diagnosed in teens and those in their 20's[2]. If left untreated, the cornea will progressively deform and vison will become increasingly distorted and may continue until effective blindness. There are however options to correct the astigmatism, halt the progression and cure the disease. In mild cases, eye glasses are prescribed to mitigate the effects of Keratoconus. As the disease progresses eye glasses may need to be replaced with rigid contact lenses specially designed for each patient's level of shape deformation. Those who are unable to use contact lenses have the option of

receiving a corneal transplant to cure the disease but this is an invasive procedure. Increasingly, riboflavin crosslinking of the cornea is used as a non-invasive treatment to halt progression and preserve vision has been of interest.

Crosslinking with Riboflavin involves three main components: a cornea to strengthen, ultra-violet light to initiate the links, and a liquid riboflavin solution as a photosynthesizer. It was first developed by Spoerl et al. in 1998. Over the years, a few small changes to irradiation time, intensity, and riboflavin solution have been made to create for a more efficient process with less tissue damage [3]. The scientific and medical communities are still uncertain where the crosslinks form in the corneal tissue nor has a quantitative effect on the stiffness been agreed upon. Most notably in the United States, the FDA recently reported that it was still uncertain if the procedure would actually be a beneficial treatment for Keratoconus [4]. Despite this, the crosslinking procedure is already approved and being used on patients in a limited number of countries in both clinical trials and approved applications. Besides clinical results, labs have been analyzing the increase in corneal mechanical stiffness provided by riboflavin crosslinks ex-vivo. In spite of this, some gaps still remain and even more are created by differing lab protocols. Compression has been touched upon by a limited number of studies [1, 5]. The main goals of this thesis are to investigate the effect of crosslinking on the compressive properties of cornea as well as to characterize the effect of using different species models.

1.2 Approach

In this thesis an unconfined compression test was utilized to determine the reaction of corneal tissue. In unconfined compression the sample is constrained laterally but is free to move in response to applied pressure in the radial direction. In the case of soft hydrated tissues, there is little radial movement by the sample, but instead interstitial fluid is able to escape the material matrix. The response to this type of test includes three phases. In the first phase the thickness of sample is decreased. This forces the interstitial liquid out and causes a sharp increase in force and is called the ramp phase. Next, in the transient phase, the thickness is held constant and the force decreases as the fluid movement slows dramatically and elastic components relax. A third phase, called equilibrium, is where with a still constant thickness, the force remains constant over time and there is no fluid movement. These compression tests were used on samples both untreated and crosslinked with riboflavin. To ensure species origin was not a factor this was done for three major species (human, porcine, and bovine.) Bovine samples were also used to test the effect of crosslinking location by treating not only the anterior, but also the posterior and both sides of the cornea together.

1.3 Expected Outcomes

This thesis is expected to add to the still little understood compressive mechanical behavior of corneal tissue by conducting unconfined compression experiments. Specifically this research work will

- determine the effect of collagen crosslinking procedure on the compressive properties of corneal tissue, and
- evaluate the suitability of bovine and porcine animal models as substitutes for studying the compressive properties of human tissues.

CHAPTER II

LITERATURE REVIEW

2.1 Eye Structure

Eyesight is important to the daily lives of most people, but typically this sense dulls or even completely fails due physical damage or physiological conditions. Despite the number of people with deteriorating eye sight, the cause of various eye conditions is still a mystery. One reason for lack of understanding is the complex, interrelated structure of the eye globe. The eye is composed of two main parts, the optical and structural

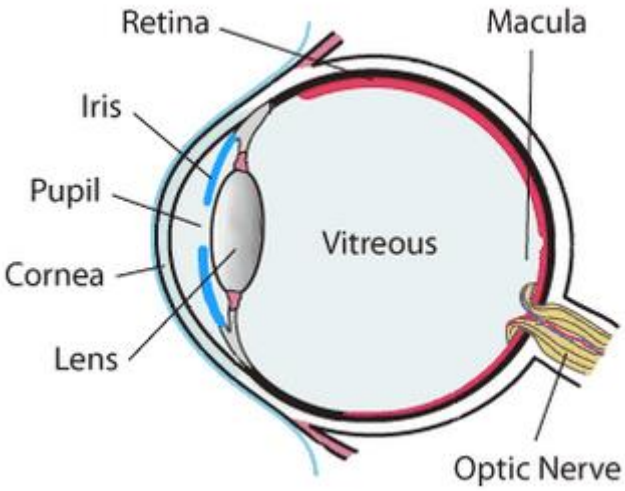

Figure 2.1. Basic eye anatomy. Source: http://heightseyecare.com/eye-anatomy/

components. The optical components must funnel the exterior light into a very precise location on the back of the retina which is then carried to the brain. The structural components are made of a flexible outer shell and an intraocular fluid that provides the pressure to keep the outer shell in shape. If the structural tissues are affected by a disorder or damage, the light may not funnel to the correct place or an increased pressure may prevent the optic nerve from functioning. An important structure that is crucial for both the optical and structural behaviors of a healthy eye is the cornea.

2.2 Cornea Structure

The cornea is the clear segment of the outer shell that both supports the shape of the eye and allows light to pass through to the interior eye. It is susceptible to scratches, blunt trauma, deformations due to ocular pressure changes, and even less understood cellular changes inside the corneal tissue itself. 5 distinct layers make up the whole of the cornea. These include, two outer protective layers the epithelium and Bowman's layer, two innermost layers the Decemet's membrane and endothelium, and between them, the thick stromal layer. While each layer is made of similar hydrated collagen material, the small compositional and orientation differences allow each to accomplish very different jobs within the eye.

The epithelium and Bowman's layer prevent both physical and bacterial damage. The epithelium is constructed of squamous cells and is essentially the protective skin of the eye. It is around 50 μm thick [6] and able to recover from physical damage in about 7 days [7]. It also provides an interface to the tear-film that keeps the outer eye moist and

prevents microbes from penetrating into the deeper layers. The purpose of the Bowman's layer is still unknown but may have in influence on epithelium healing speeds [8]. Although it is present in humans, the Bowman's layer is not found in all species [9, 10]. Cattle possess a thin 5 μm Bowman's layer but pigs lack this layer entirely.

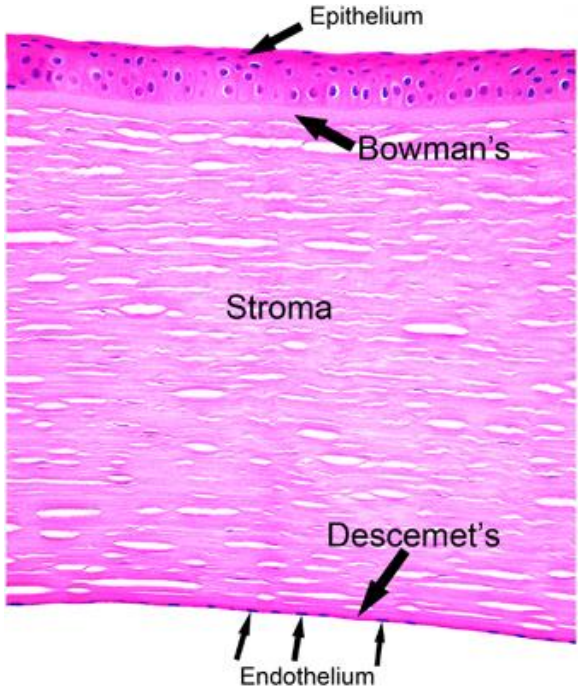

Figure 2.2 Corneal layer arrangement. Reprinted by permission from Macmillan Publishers Ltd: [Eye] Meeney, A and Mudhar, HS, Histopathological reporting of corneal pathology by a biomedical scientist: the Sheffield Experience, copyright (2013)

Descemet's membrane along with the endothelium allow the transfer of nutrients and maintain the constant hydration of the cornea. Although the Descemet's membrane is only 10 μm thick, proper hydration levels (needed for clarity of the corneal stroma) are dependent on it [11, 12]. The endothelium uses a pump-leak method to keep the correct hydration level. The cells are also unable to regenerate so a deficiency of them may lead to excessive corneal swelling and loss of sight [13].

The stroma constitutes nearly 85% of the corneal thickness and provides structural support for the eye globe due to its strong corneal matrix composition [14]. At the microscale the cornea is a hydrated mesh of collagen fibers and proteoglycans much like other semi-flexible bodily tissues such as cartilage and ligaments. The most common collagen (type I) is found in bundles called fibrils along with sparing amounts of type V [15]. These long bundles extend continuously from one side of the limbus to the next and are the main support structures. Attached to these collagen fibrils are proteoglycans which are thought to assist in spacing the fibrils and provide anchors to nearby fibrils [16]. Collagen type VI is also found interspersed between the fibril spaces and assist the proteoglycans in securing the load bearing fibrils [17]. Fibrils in the human cornea have an average radius of 15 nm with a slight increase as aging occurs and in the central cornea have an interfibrillar spacing of 57 nm [18, 19]. In the next level of organization, multiple fibrils are located in sheets called lamella. The arrangement of lamella have been found to vary by species. In humans the primary orientation in the central region consists of lamella layers perpendicular to those above and below with the pattern morphing into a ring type pattern as the limbus is approached [20]. In comparison, the lamella

arrangement of cattle are mostly diagonal and porcine eyes are arranged in concentric rings like those near the exterior of human corneas [20].

2.3 Riboflavin Crosslinking

Little is known about how or why Keratoconus starts but the symptoms are blurred vision due to a defamation of the corneal shape. This change in shape is accompanied by a thinning of the corneal thickness as well as a corresponding decrease in elasticity. If untreated this condition will continue and vision will progressively decrease. An accurate number of people affected by this disease is difficult to determine due to slow symptom progression but studies suggest at least nearly 54 people out of 100,000 are affected [21]. The main treatment depends on the severity of the disease state with eye glasses used for very mild cases, specialty contact lenses for mild or moderate, and for the most extreme cases, a complete corneal transplant.

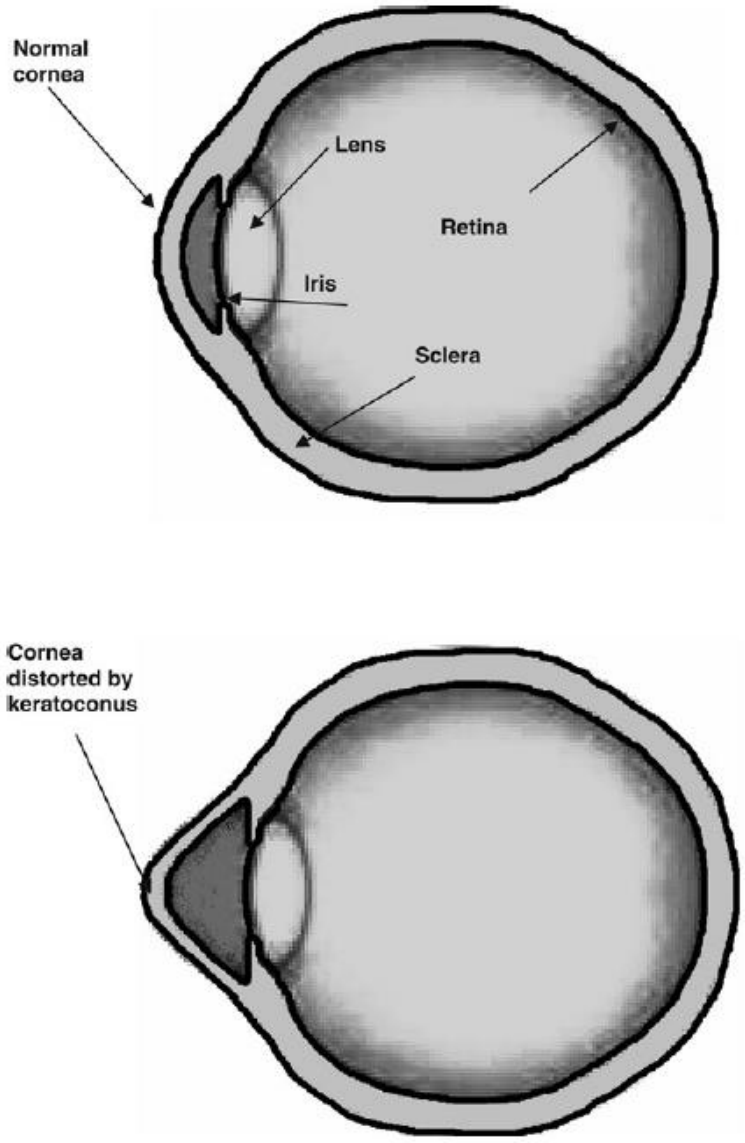

Figure 2.3. Examples of a healthy and Keratoconus affect corneal shape [22].

Relatively recently, corneal crosslinking by way of riboflavin-ultra violet light has offered hope for a noninvasive if not permanent way to halt Keratoconus. The process requires a light source to provide the energy to form the crosslinks and a photosynthesizer to increase efficiency of light absorption. Through trials the ideal combination was found

to be UVA light at 370 nm wavelength providing an intensity of 3 mW/cm^2 for 30 minutes when using riboflavin-5 phosphate as the photosynthesizer [23, 24]. Although it causes mild discomfort for patients the epithelium is typically removed in order to increase riboflavin absorption and provide 5 times the effectiveness of the procedure [25]. Once complete the procedure prevents Keratoconus from worsening for years [26].

Zhang et. al. used chemical analysis of in-vivo and ex-vivo samples to suggest that the most common locations of crosslinks are between collagen and proteoglycan as well within a collagen fiber [27]. Hayes et. al. used x-ray scattering analysis to support the findings of Zhang et. al. in that crosslinks were likely to form within a collagen fiber. However, they also used lack of differences between crosslinked and untreated groups during swelling/dehydration results to determine that the likelihood of widespread collagen to proteoglycan linkage improbable [28]. The results in Hayes et. al. were in favor of crosslinks forming in 4 locations, the previously mentioned intramolecular case, between branches of one proteoglycan, between two proteoglycans, and between two collagen molecules at the surface of the fibril.

2.4 Mechanical Tests

Various types of mechanical testing have been done on both the crosslinked and untreated corneal tissue. The overall goal is to determine how certain conditions affect the tissues. Once it is known how they behave, various models can then be constructed that predict the behavior allowing for a non-physical method of testing. Also, by comparing the untreated and crosslinked samples, more can become known about where

the crosslinks form and how they benefit patients who receive this treatment. Due to the complex substructure of the tissue a wide range of tests are beneficial to achieving a full understanding of behavior.

Since the eye in its natural state is a full globe, there is benefit in studying the whole or partial corneal scleral region. In these experiments, called inflation tests, fluid is injected to the globe to control the pressure via a small incision in the posterior sclera or as a securely clamped half sphere with a posterior fluid chamber [29, 30]. As the interior fluid pressure is increased the tissue stretches and deformation is recorded. Though it attempts to mimic natural conditions, the samples still must be pinned and experience non-natural constraints. The method also requires specially designed testing equipment. Furthermore, there is also no mathematical relationship to translate the results from strip deformation to equivalent in vivo stresses [31].

Since the structure of the cornea is essentially a three dimensional mesh of collagen fibrils with fluid in-between, the ability to swell is thought to have a possible relation to crosslinking. An initial study showed that there crosslinking did cause a slower swelling rate when crosslinked on the anterior side [32]. However the results were not able to be replicated by another study where no difference in swelling rate was found [28]. The discrepancy may be attributed to differing structural composition of the stroma with changing thickness or residual crosslinking fluid which contains chemicals that prevent swelling. Furthermore some clinical trials for bullous keratopathy, a disease that causes excessive swelling of the cornea, showed that the decrease in swelling only lasts for a few

months while the strengthening benefits for treatment of keratoconus last for years [33, 34].

Tension is the most widely studied behavior of corneal tissue. The main reason for this is that the eye is essentially a pressure vessel which converts internal pressures into forces that are oriented through the material instead of tangentially. In two dimensions, the equivalent force direction would be tension so this is a convenient way to estimate if what is being tested could have an impact on an actual eye.

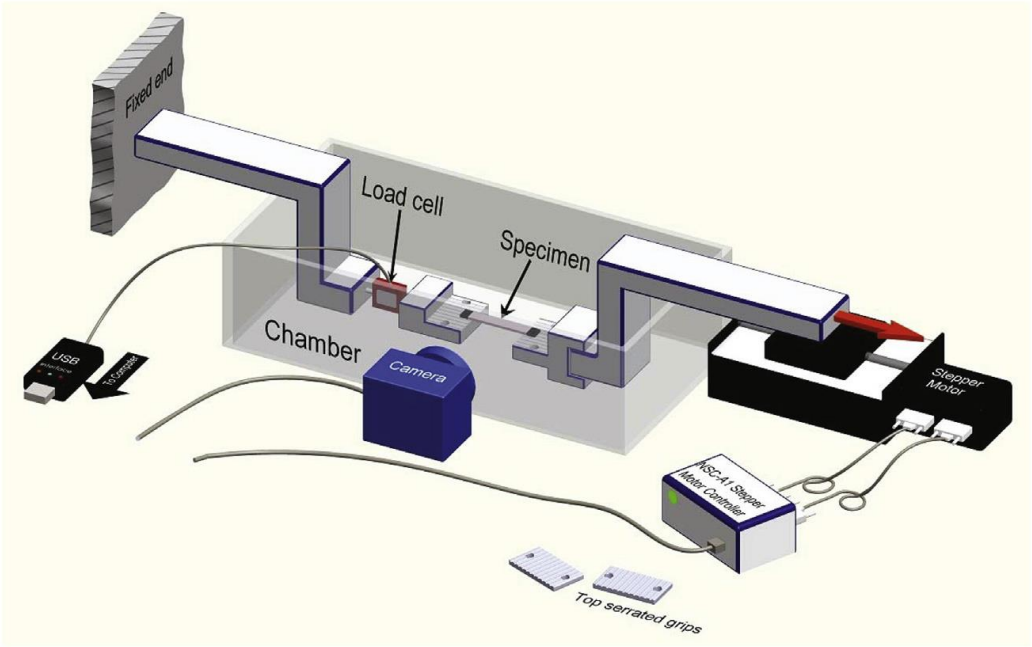

Figure 2.4. General tension experiment layout [35].

Another reason for the popularity is that the testing equipment needed is generally accessible for most laboratories. For this type of testing a corneal strip is excised and clamped between two mounting locations then typically immersed in a bathing solution to prevent dehydration. While the setup is the same, many other factors are varied from laboratory to laboratory. It has been found that hydration, bathing solutions, temperature,

strain rate, and species type all play an important factor in the results obtained but are not always controlled or even measured [35-40]. Due to the variation in protocol the results for the tensile stiffness have a rather wide range of results.

Although the intraocular pressure mainly acts on the tissue in a tensile direction, various forces such as poking or rubbing produce a compressive force on the cornea. As of now, only a limited number of studies have been done in compression. It has been found that a

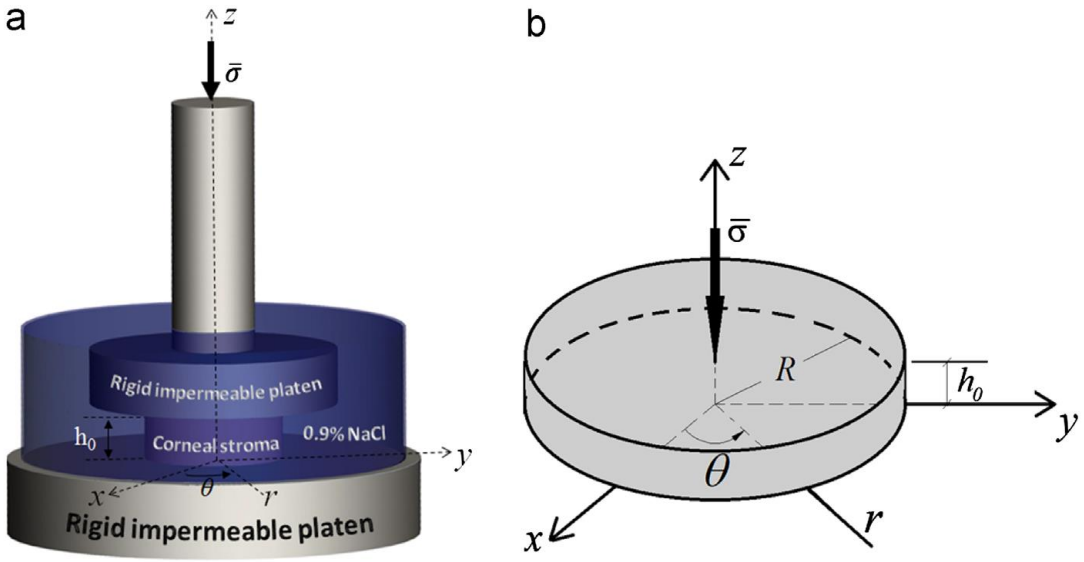

Figure 2.5. a) Basic unconfined compression testing set up. The circular sample is compressed between two ridged plates while a saline solution maintains hydration. b) A representation of a sample with the measured stress (σ) and thickness (h) depicted. From source [1].

cornea sample under compression will show an initial stiff response characterized by an increase in stress-time slope due to the pressure of the fluid exiting the stromal matrix. After compressing if a constant thickness hold phase is carried out, the sample exhibits two more distinct phases (transient and then equilibrium) as the fluid flow and flexible structural components settle [1]. With such behavior it has been concluded that in

unconfined compression, corneal stroma behaves very similarly to that of other porous and elastic tissues.

Characterization of elastic moduli and permeability coefficients have been found to change in response to decreasing thickness. This is to be expected as the change in thickness is directly related to hydration and lubrication of the collagen fibrils [41]. A slightly different method was used by Kontadakis et. al. to investigate immediate compressive effects of CXL treatment. In their study a 1mm diameter spherical ball was pressed against the cornea in comparatively large increments of 20 μm for both crosslinked and untreated samples. Their results shows no statistical difference between either of the groups and the researchers suggested that the crosslinks form within a fibril or between adjacent fibrils and not between the lamella layers [5].

Other less prominent mechanical testing types include impact, air puff, AFM, nano-indentation, fracture toughness, and shear [42-45]. There are also non-mechanical tests such as enzymatic degradation, hydration-thickness relations, and various microscopy studies [28, 46, 47]. These are being used to look at the impact of fibril arrangement, proteoglycan and crosslink bond stability, and interfibrillar spacing among other things that are directly related to the mechanical stiffness. Combine this with the computational models and finite element methods and there is an astounding amount of work being focused on furthering the understanding of riboflavin crosslinking and corneal mechanics [22, 45, 48].

CHAPTER III

MATERIALS AND METHODOLOGY

3.1 Sample Preparation

In total, tissue from three species; human, swine, and bovine was used. Preparation typically differed mostly in the use of bathing solution, corneal button diameter, and existence of crosslinking treatment. Which combinations were used depended on the nature of the tests and not the species. Because human tissues have more regulations and medical uses; however, the basic preparation method does differ.

Human corneal-scleral rings, unsuitable for use in corneal transplants, were obtained from the Cleveland eye bank. The average age of the donors was 59.7 years with a standard deviation of 10.0 and a range of 45 to 75 years. These samples came sealed individually in corneal viewing chambers with an immersion fluid of Optisol-GS. Samples were obtained and maintained at low temperature in order to extend preservation. Samples were removed from refrigeration just before preparation for use where the sample was gently scrapped with the dull side of a curved surgical scalpel to

remove excess epithelium and endothelium. The sample was then blotted gently with a Kimewipe to remove excess Optisol-GS and air dried until the sample reached an approximate thickness of 550 μm. At this thickness a corneal button was removed with a 5 mm diameter biopsy punch which was then allowed to rehydrate in Optisol-DS to thickness of 850 μm in order to be well above in-vivo thicknesses.

Preparation for bovine and porcine samples were typically identical. Enucleated eye globes were obtained from a local butcher shop and transported in a cooler back to the laboratory where they were frozen whole at -20^{0}C until use. Prior to use, the globe was allowed to fully thaw at room temperature. The epithelium was removed with the dull side of a curved surgical scalpel and an incision created in the sclera just below the limbus. Surgical scissors were then used to extract the corneal-scleral section and the endothelium removed with the dull scalpel. The corneal button was then removed with a biopsy punch.

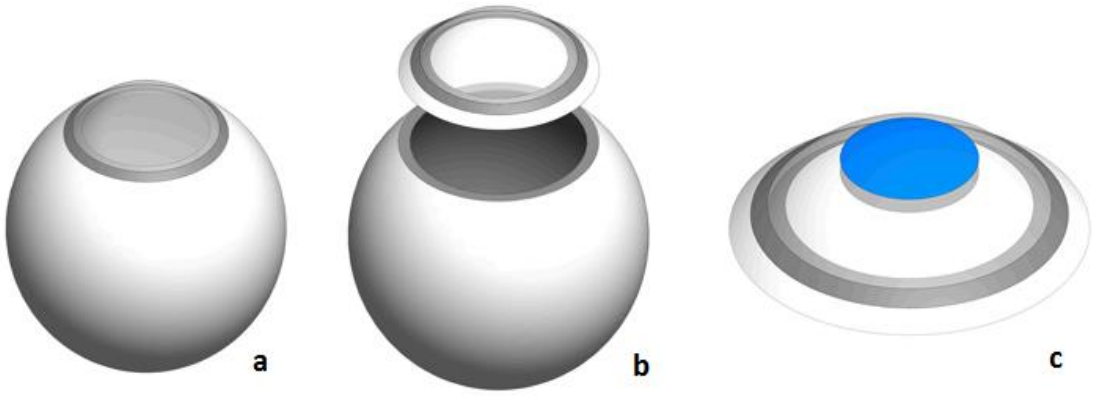

Figure 3.1. a) Whole eye globe. b) Initial corneal-scleral ring dissection of the sample. c) Final dissection of the corneal button from the central cornea area highlighted in blue.

3.2 Bathing Solutions and Measurements

One small component of this project involves the use of different bathing solutions during testing in order to obtain information about the mechanics of fluid reabsorption in compression. 0.9% NaCl solution is commonly referred to as normal saline solution when used intravenously in the medical field or in commercial eye drops. The osmolality of the 0.9% saline is nearly identical to conditions found in healthy bodily fluids such as blood and tears. This in combination with its low cost and ease to create have made it a commonly used bathing solution for previous tests.

Balanced salt solution (OBSS) (ALCON laboratories, Inc., Texas, USA) is primarily used in medical procedures in place of saline solution. It is a sterile solution composed of sodium choloride as well as other chemicals beneficial to the eye such as potassium chloride. The main drawback of this solution is that it cannot be mixed in house but must be bought at a higher cost than the previously mentioned saline solution.

Optisol is the third main bathing solution investigated. It is a solution created specifically to preserve corneal tissues prior to transplantation. It is a complex mixture of water, pH buffers, anti-swelling, antibiotics, and vitamins tailored for eye bank use. It also contains a pH indicator (phenol red) giving it a rosy red color that brightens to pink with increasing pH that may indicate a bacterial infection of the tissue. Since the human tissues were obtained from eye banks, they had been immersed in Optisol-GS which prompted us to investigate its possible effects on mechanical behavior.

3.3 Crosslinking Procedure

Crosslinking involves three components. A riboflavin solution, corneal-scleral section, and an ultra-violet light. The 0.1 % riboflavin solution used is made in lab with 10mg of riboflavin-5-phosphate sodium salt dehydrate (Alfa Aesar, Massachusetts, USA) with 20% dextran T-500 (Spectrum, California, USA). The 20% detran solution prevents over absorption of liquid inside the cornea which would run the risk of over extending and breaking structural tissues. The riboflavin is the key photosynthesizer which allows more of the UVA light to penetrate the cornea and create crosslinks. The preparation of the corneal-sclera section is discussed in the beginning of this chapter. After removal porcine and bovine sections were immersed in the riboflavin solution for 2 hours. Since the dextran naturally prevents swelling, the thickness changes with immersion time. From previous trial runs it was found that a time frame of 2 hours allowed the samples thickness to reach equilibrium and allow for a common crosslinking thickness. The procedure for human eyes was slightly different due to their thinness and age. These tissues, instead of immersion, had riboflavin solution applied every minute for 5 minutes prior to UVA irradiation. After absorption of the riboflavin crosslinking was begun. The corneal-scleral section was placed on a wooden half sphere in order to mimic the natural curve and prevent pooling of riboflavin solution. The sample was then placed under two infrared LEDs with a wavelength of 365 nm which at a distance of about 16-20 mm have an intensity of 3 mW/cm^2. The ultraviolet light was applied for 30 minutes with more riboflavin drops added to the surface of the sample every 5 minutes. Unless otherwise specified, the anterior side of the cornea was facing the LEDs during crosslinking.

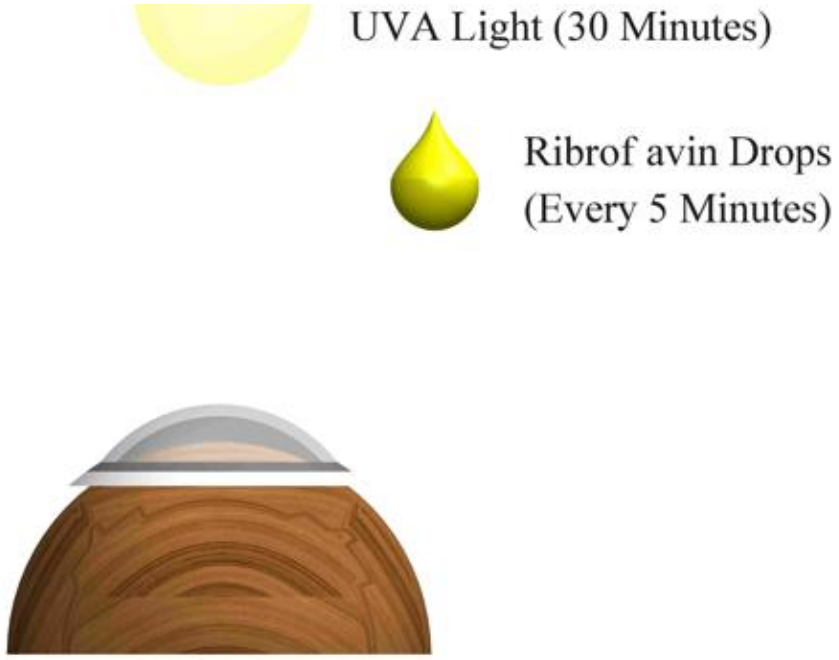

Figure 3.2. Crosslinking of the corneal-scleral ring. UVA light and Riboflavin are applied to the central region of the corneal-scleral sample.

3.4 Testing Procedures

Compression tests are the primary focus of these studies. The majority of compression and all tensile tests were conducted on an RSA-G2 material testing device (TA Instruments, Delaware). Some bovine samples were tested using a Discovery Hybrid Rehometer (TA Instruments, Delaware) in order to utilize a temperature control system. The testing procedure was nearly identical between machines with the only differences due to the nature of investigations being done.

3.4.1 Compression

The thickness of cornea naturally varies between individuals so a preconditioning phase was carried out in order to compensate. A set target stress was selected and a target force was calculated by using the measured diameter of the specific sample. During preconditioning, a sample was compressed and then allowed to relax for 30 minutes. These two steps were carried out until the force at the end of the relaxation phase matched the calculated target force. The thickness directly after preconditioning was considered to be the initial thickness. Preconditioning also has the advantage of ensuring good contact and eliminating any air bubbles between the sample and the compression plates.

During the test, percent strain was calculated from the tare thickness and were typically increased in increments of 5%. A compression phase was always immediately followed by a relaxation phase whose length depended on the strain rate obtained and diameter of the sample. The spongy nature of the cornea causes force relaxation even though the devise causing the initial load has not moved. Due to this both a maximum and a relaxation force is beneficial to record. The maximum force is recorded just as the sample reaches the target strain and equilibrium is an average of the last 20 seconds of the relaxation phase. Typically the compression phase is a ramp with a linear displacement of 0.15 μm/s although a limited number of instantaneous compression were conducted where the full strain was obtained in a fraction of a second.

3.5 Data Analysis

A one way ANOVA test was used to compare the differences between different groups and between different strains. A level of significance of 0.05 was used in all instances. To remove outliers, the Modified Thompson Tau method was used.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Species Variation

Corneas of different species differ both macro and microscopically. These differences include not only height to width ratios and differing thickness, but also in vivo hydration, and collagen layout arrangements [28, 49]. The sections below will summarize the specific testing protocols and results of dual species compression. Each test was run with an untreated sample in a submersion chamber containing Optisol-GS, a tare load of 2.5 kPa, and a diameter of 5 mm.

4.1.1 Porcine and Bovine

Both porcine and bovine groups originated from the same butcher shop and were transported, stored, and prepped in the same manner. Samples were all dissected and then allowed to absorb 0.9% NaCl in order to reach a greater than in-vivo thickness prior to placing in the Optisol-GS submersion chamber. Before initiating the preconditioning

phase bovine and porcine both registered a thickness of 1100µm. 7 samples were included in the bovine testing group and 6 in the porcine.

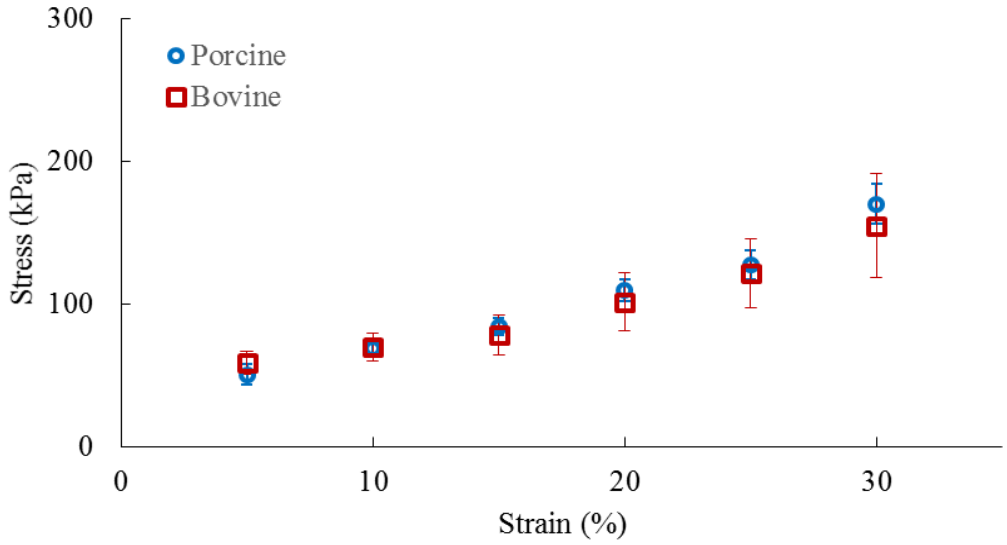

Figure 4.1 Average maximum stress vs strain % results between porcine and bovine untreated samples.

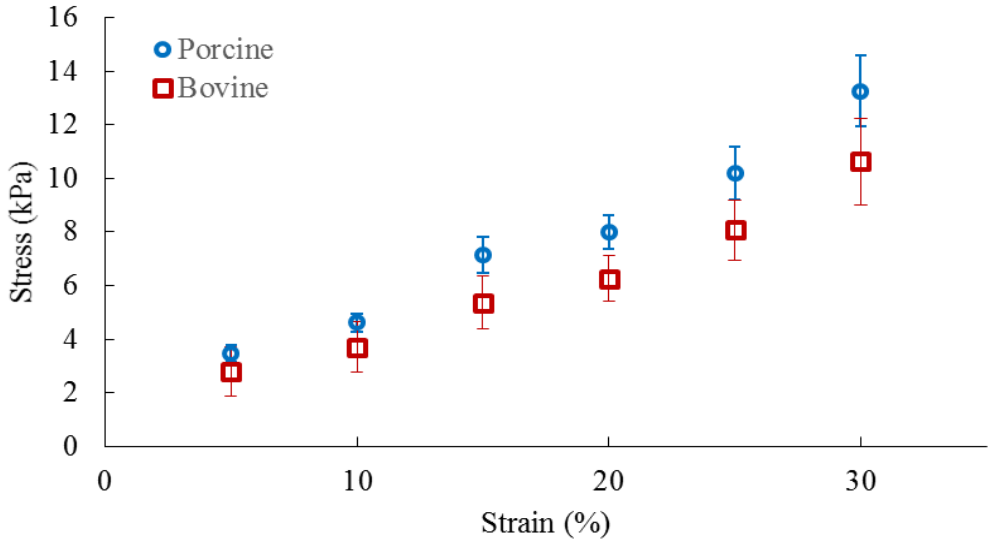

Figure 4.2 Average equilibrium stress vs strain % results between porcine and bovine untreated samples.

4.1.2 Porcine and Human

Human samples were received in Optisol-GS to aid in preservation but once the button was punched they were allowed very briefly to swell in 0.9% NaCl solution. Before precondition the average human thickness was near 750µm compared to the porcine initial thickness of 1100µm. The human testing group included a sample size of 5 and the porcine 6.

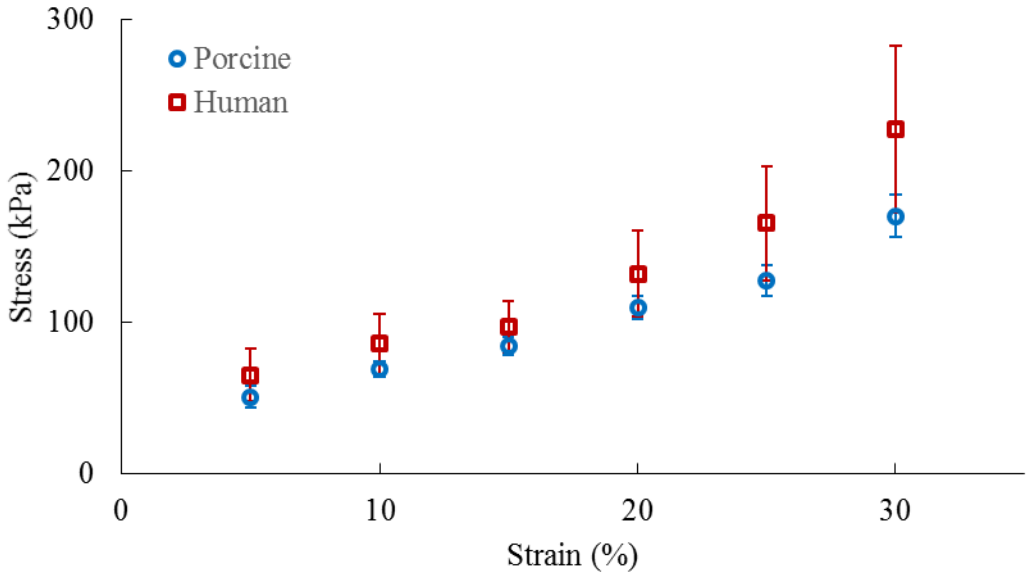

Figure 4.3 Average porcine vs. human results for maximum conditions.

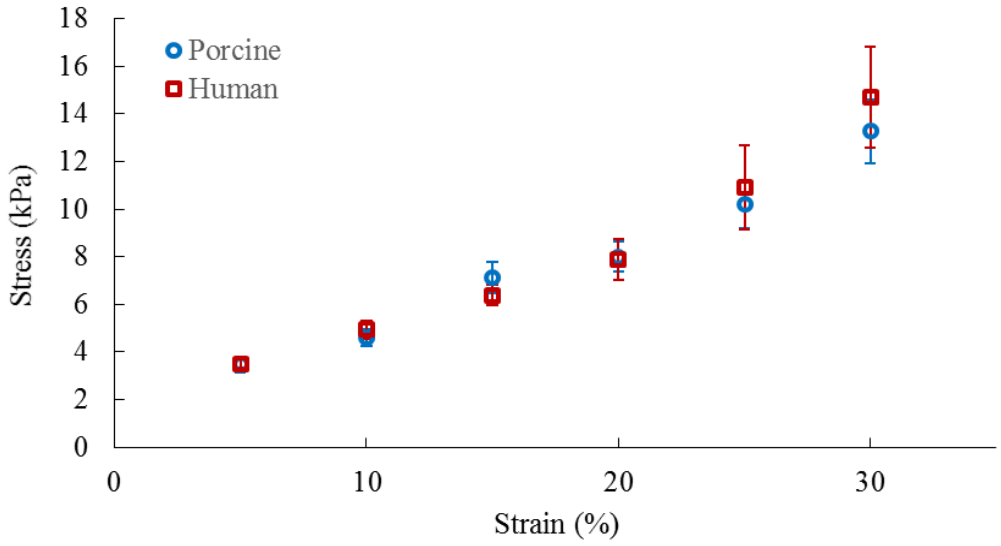

Figure 4.4 Average porcine vs. human results for equilibrium conditions.

4.1.3 Human and Bovine

The set up for this grouping is much the same as the human vs. porcine above. Bovine had an initial thickness of 1100µm and a sample size of 7 while human had an initial thickness of 750µm and a sample size of 5.

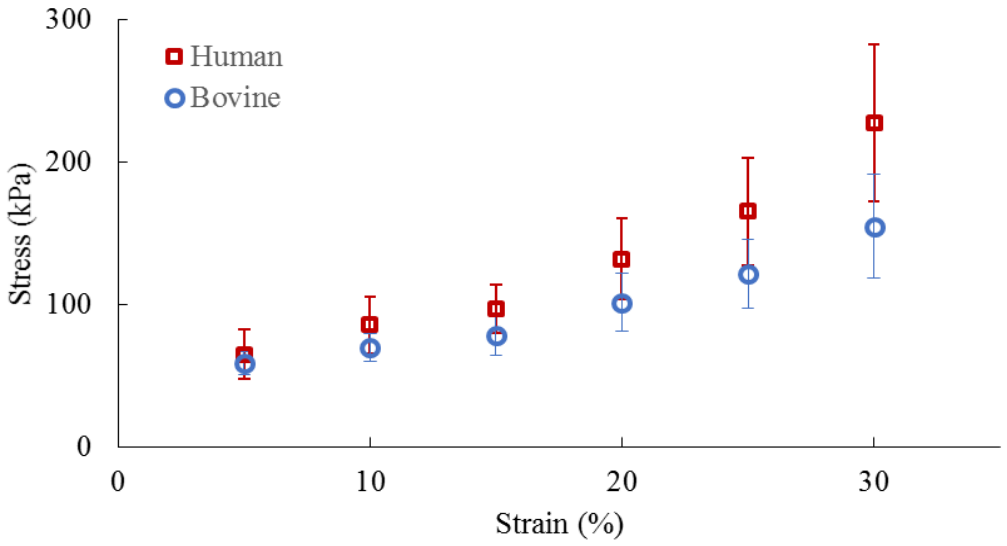

Figure 4.5. Average human vs. bovine results for maximum conditions.

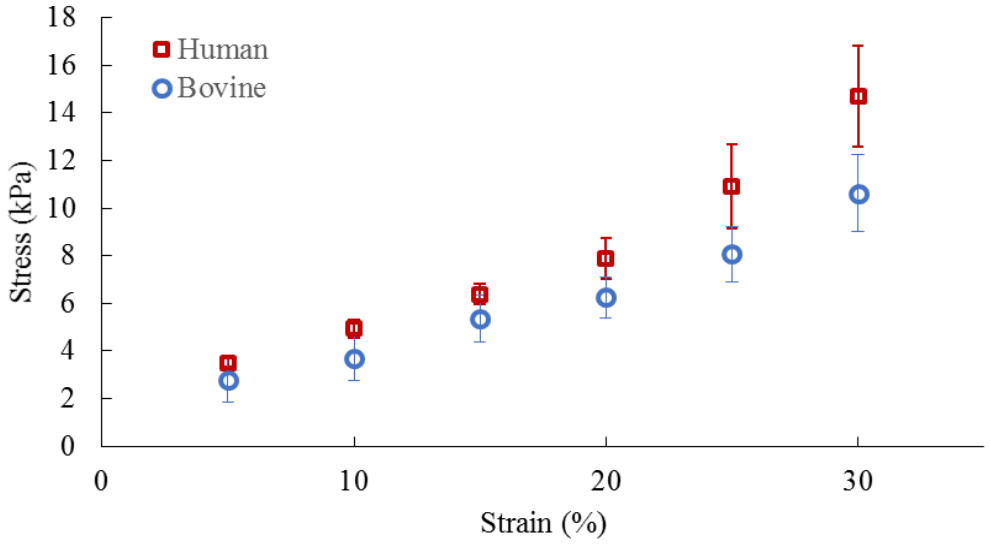

Figure 4.6. Average human vs. bovine results for equilibrium conditions.

4.1.4 Species Variation Summary

The first thing of note in the species comparison is that the human samples show a much stiffer response than that of either porcine or bovine. This aligns with the results found in previous tensile literature where the porcine group was at least slightly less stiff both in the untreated and crosslinked case. An average ratio of maximum to initial stiffness for human samples within the 65-79 age was 9 as compared to that of the porcine which recorded as an average ratio of 5 [40]. Furthermore an increase in stress from untreated to crosslinked samples for porcine was recorded as 71.9% for porcine and a much larger 328.9% for human samples [50]. Another thing to consider when comparing the results on eyes originating from human cadavers is the donor's age. While animal tissue is widely available at younger ages, the vast majority of human tissue originates from organ donors after death typically at an advanced aged. Previous studies have shown that the arrangement of the stromal fibers changes with age and that the diameter of fibrils increases [18]. A difference in the average ratio of final to initial stiffnesses varied from 11 for an age range of 50-64 years to a 5 for a range of 80-95 years [40]. An increase in the diameter of the loadbearing fibers would lead to a stiffer material and the experiments of Elsheikh et al. show such an expected rise in compressive properties as age increases [51].

Although both are commonly used as a substitute for human corneal tissue, porcine and bovine behaviors are not often compared. This study found that between the two, the porcine group consistently showed a higher compressive stress. It should also be noted that the average tare thickness of the bovine group was approximately 100μm less than

that of the porcine group despite having a larger in vivo thickness. This is likely due to the bovine cornea having a naturally higher hydration than many other species including porcine and human as found in Hedby et al. and Hatami-Marbini et al. [49, 52]. With a higher hydration the bovine cornea can be expected to show a less stiff response at higher thicknesses due to interstitial fluid being able to flow out in unconfined compression. In order to further understand both the compressive behavior of the viscoelastic cornea and the effect that riboflavin has more investigation is needed.

4.2 CXL Effect

4.2.1 CXL Effect Current Results Summary

Compressive tests for both untreated and crosslinked sample groups were run for three different species. The crosslinked groups were treated with the riboflavin and UVA as discussed in chapter 3. The crosslinking produced was applied to the anterior side of the cornea unless otherwise noted such as in the bovine group. In that bovine study the locations of the crosslinks are further specified. Due to the differences in in vivo thickness as well as other physiological species based variations the results are analyzed in species specific sections.

4.2.2 CXL Effect on Bovine

In this procedure a bovine cornea was placed in the untreated group or crosslinked on the anterior, posterior, or anterior and posterior sides. The button diameter was set at 8mm and a tare stress of 3.5kPa was used an initial before preconditioning thickness of 1200μm. Each treatment group has a sample size of 10 and the tests were conducted at a steady temperature of 37 °C.

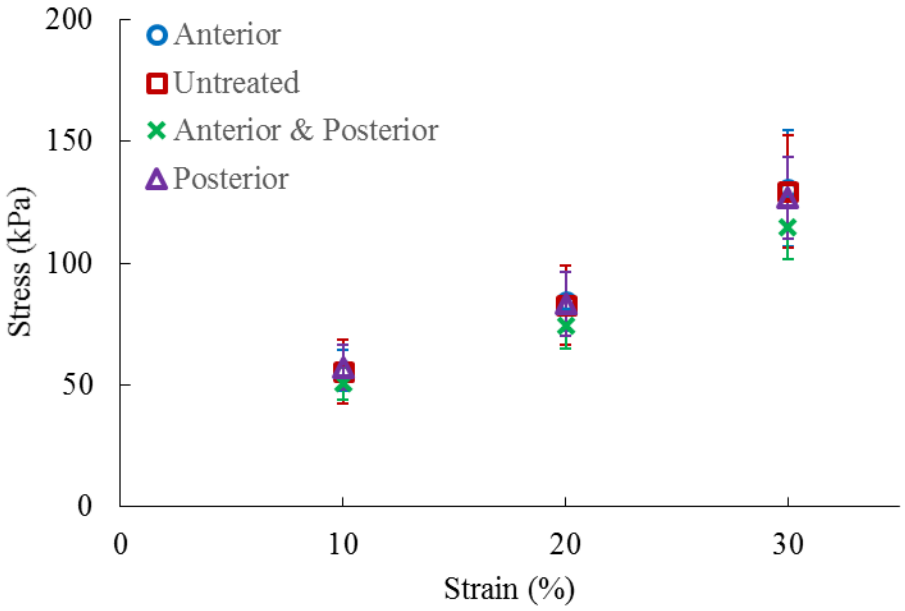

Figure 4.7. Average maximum results of crosslinking when preformed on either the anterior or the posterior and on a combination of both anterior and posterior as compared to an untreated sample of bovine cornea.

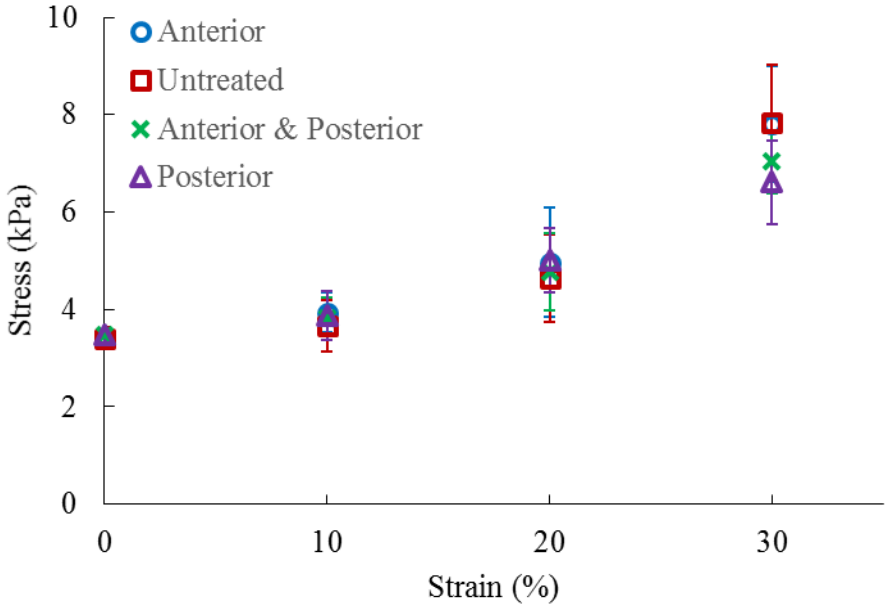

Figure 4.8. Average equilibrium results of crosslinking when preformed on either the anterior or the posterior and on a combination of both anterior and posterior as compared to an untreated sample of bovine cornea.

The results above show little difference between the sample groups. An ANOVA test sound no statistical difference between untreated and any CXL treatment except at the 30% equilibrium for the posterior group. This issue is still under investigation and as such the results may still change.

4.2.3 CXL Effect on Porcine

A crosslinked group (n=9) and an untreated (n=7) were tested at a tare stress of 3.5 kPa, with a diameter of 6.5 mm, and an initial thickness of 1200µm. These groups were also tested using a 0.9% NaCl bathing solution. Temperature was not monitored but remained equivalent to the ambient.

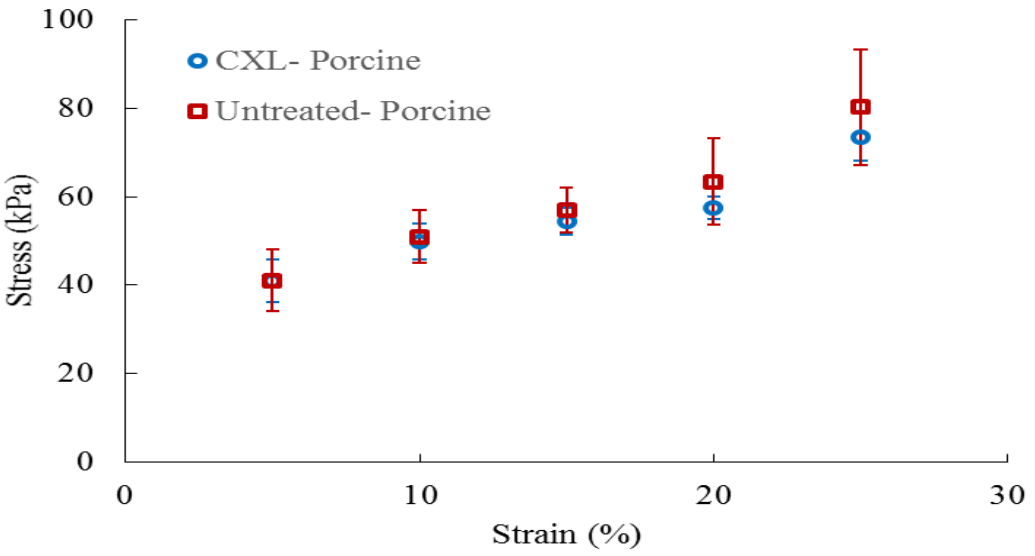

Figure 4.9. Average porcine crosslinked vs. untreated results for maximum conditions.

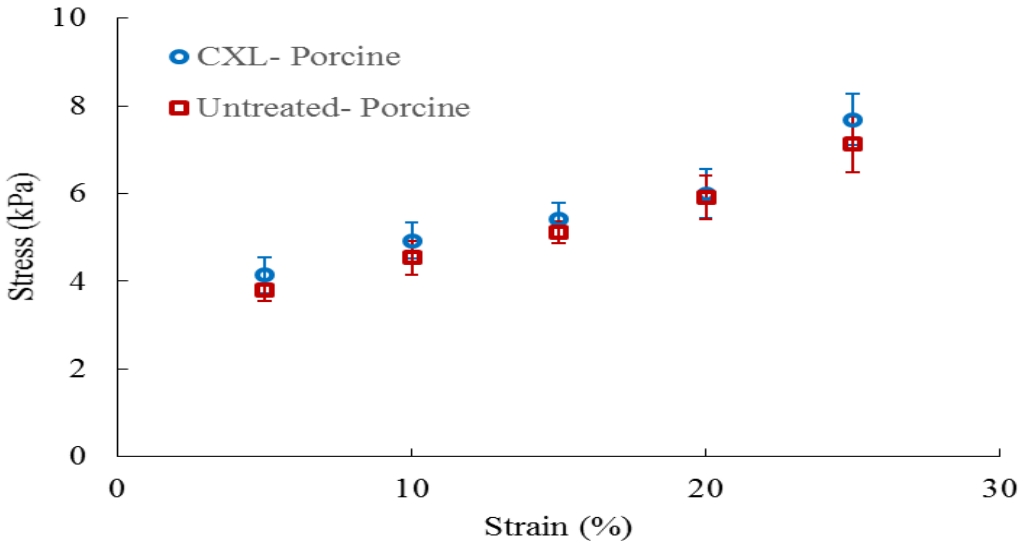

Figure 4.10. Average porcine crosslinked vs. untreated results for equilibrium conditions.

The ANOVA tests detect no significant difference between crosslinked and untreated at any strain level.

4.2.4 CXL Effect on Human

The untreated (n=5) and crosslinked (n=5) groups were tested in Optisol-GS with a tare stress of 2.5kPa, and diameter of 5mm. There is a significant difference in one location, the 5% equilibrium stress.

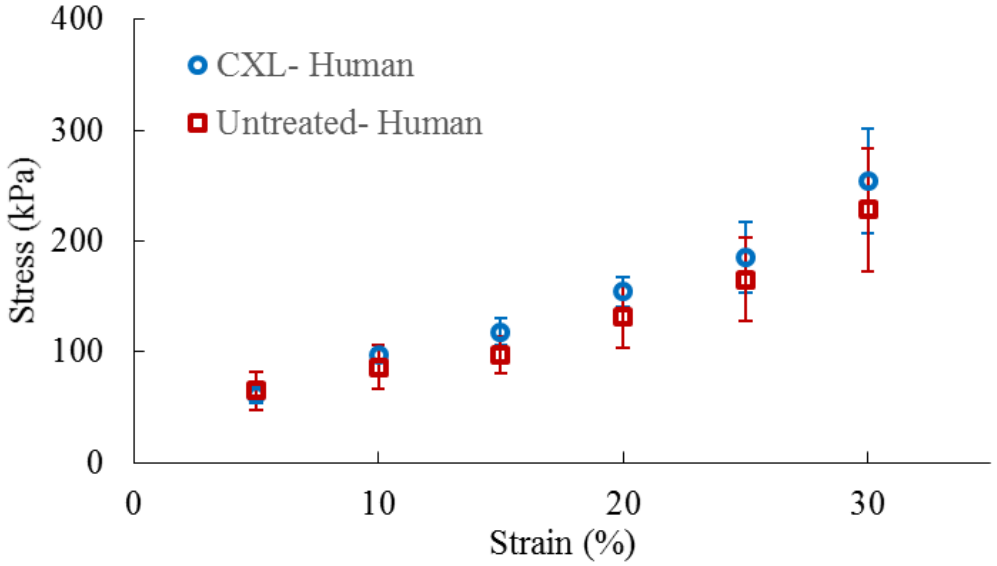

Figure 4.11. Average human crosslinked vs. untreated results for maximum conditions.

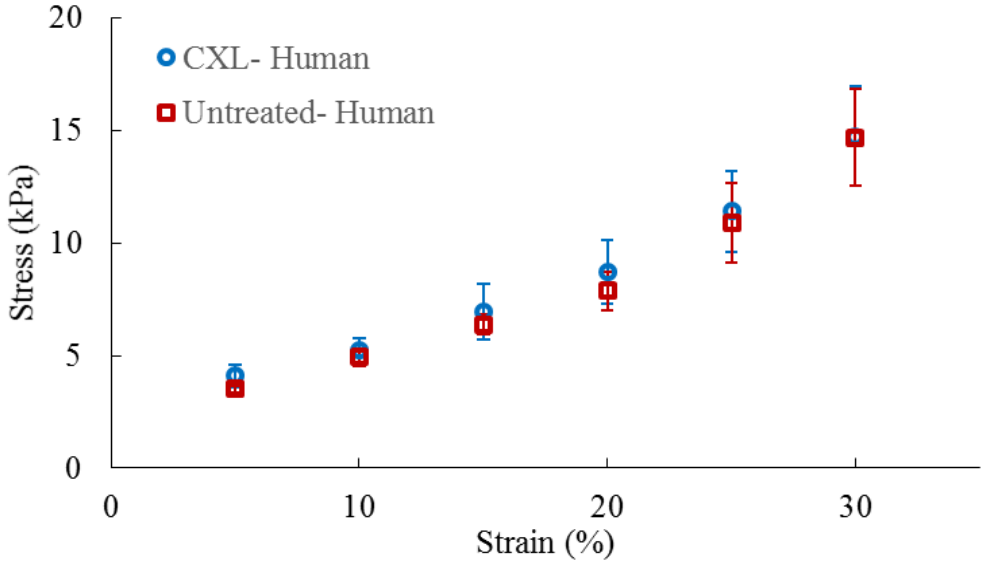

Figure 4.12. Average human crosslinked vs. untreated results equilibrium conditions.

4.2.5 CXL Summary

Significant difference between crosslinking and untreated groups was found at only a few locations regardless of species. This suggest that if the crosslinking has an effect on the compression stress that it is not reliably measured. In a compressive test utilizing a 1mm diameter ball as a force applicator found that there was not a measurable difference between compression behaviors of porcine corneas when treated with riboflavin crosslinking [5]. Conversely an atomic force macroscopy based approach for determining the compression behavior found that the anterior region possessed increased compressive properties [53]. It may be that with a microscopic approach small areas of the fibrous collagen network are undergoing tensile behavior or that the macroscopic scale behavior differs from that of the microscopic.

CHAPTER V

CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The results presented and discussed in the previous chapter will now be wrapped up. Due to the mechanisms in cornea compression being relatively unstudied, initial studies have been conducted in order to further understand parts of the compressive behavior. These preliminary studies should be further explored in future work.

Between the different species many different behaviors were found. Using the standard preconditioning to a tare force method, the porcine model was found to be a closer match to human samples than bovine was. Between porcine and human the equilibrium results were nearly identical. The maximum behavior showed a divergence as the strain increased. This suggests that porcine might be a good model for both equilibrium analyses and low deformation maximum behaviors. This presents future investigators with an option of using either porcine or bovine tissue models.

Statistical testing (ANOVA) showed that there were very few strain levels where the crosslinking procedure could be seen to have an effect on the compressive mechanical properties. The presence of even these few strain levels where crosslinking was found to increase the compressive stress shows that there is only a slight affect in compression. Since there were very few actually statistically proven it suggests that in compression, the improvement is a very delicate thing to measure accurately. This fits with the current state of compressive research where nanoscale methods such as AFM are able to consistently detect a compressive force increase with riboflavin treatment but macroscale procedures, like those presented here, show little or no effect [1, 5, 41].

5.2 Future Work

Since some of the samples needed to be maintained below the freezing point a storage medium was needed. Storage medium has the possibility of completely infiltrating the matrix and then affect the results of the experiments; therefore, one possible extension of the current study is to investigate the effect of storage medium. Within these experiment sets Optisol-GS as a storage medium for the human samples might also have influenced the compressive behavior of the human results. Preliminary testing on the effect of bathing solution during compression of cornea shows that that regardless of the species Optisol-GS produces a stiffer response for porcine cornea. Even though the bovine and porcine samples were immersed in the Optisol-GS during testing, it is still possible that some residual 0.9% NaCl was inside the corneal matrix. Due to the limited ways in which human tissue are available factors such as this should be investigated more fully with human tissue in order to determine the effect on results. We have conducted some

preliminary studies relating to this idea but a more controlled and direct investigation should be done.

Another factor is the bathing solution during the testing procedure. Literature on the effects of bathing solution on tensile behavior have shown a wide range of effects [35]. If compression is to become increasingly studied the bathing solution effects would be better to know sooner rather than later as differing protocol between labs have the prospect of distorting results. This study should be conducted on human as well as bovine and other prominent animal tissue models such as rabbit. We conducted a preliminary study that shows a wide range of results even with a limited number of bathing solutions and species. For a full analysis a much larger number of bathing solutions should be investigated.

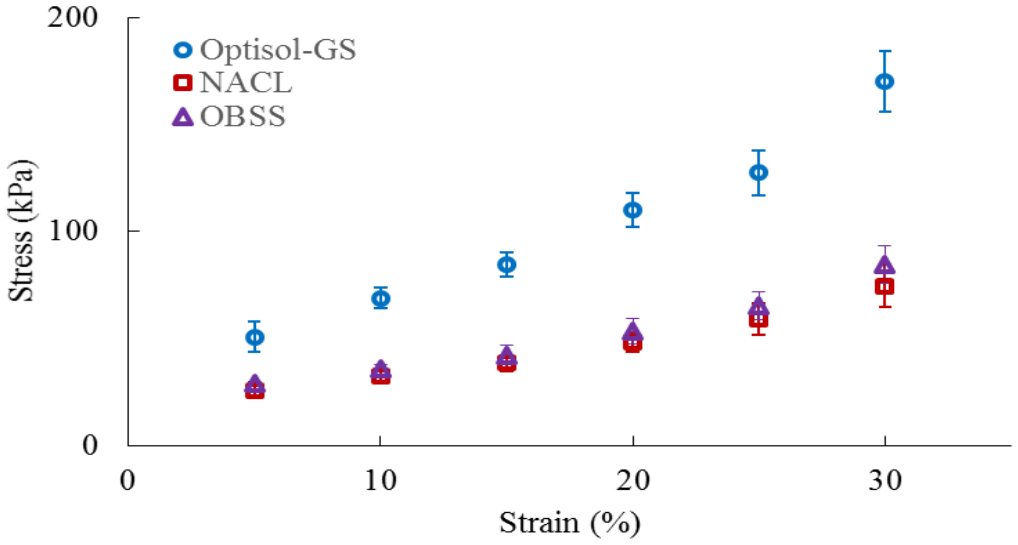

Figure 5.2.1. Average results of maximum behavior when bathing solution is varied.

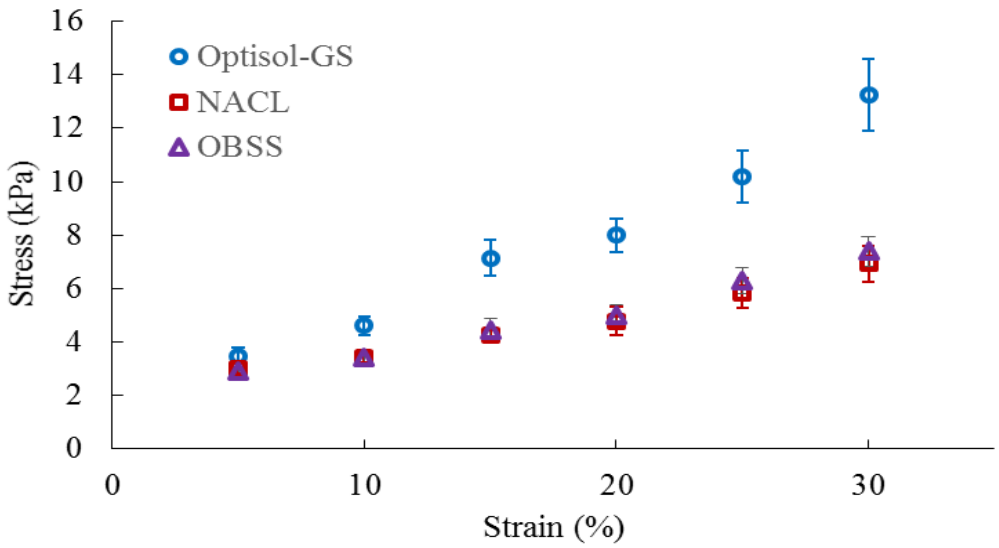

Figure 5.1.2. Average results of equilibrium behavior when bathing solution is varied.

Also closely related to this study, the decision to use the stress-strain or the stress-thickness relation also needs to be investigated. In most of the compressive tests on cornea a tare stress has been added in order to account for natural thickness variation between individual samples of a similar species. Different species have differing behaviors including hydration profiles, in-vivo thickness, and as such the stress vs. thickness behavior does differ. Due to different needs in the approach of data analysis stress-thickness behavior should be investigated further.

The difference between the strain based and thickness based relationships is likely due to the structural differences in the fibril arrangements and associated in vivo hydration of the stromal layer. Understanding which difference or combination of differences lead to a certain effect on the relationships will allow the behavior of cornea in compression to be further understood. It will also allow researchers to more accurately predict how they can

expect an animal model to differ from human behavior in future investigations. To accomplish this using buttons cut from different locations on corneas from the same species can be compared to each other due to their differing collagen layout. The initial hydration of the samples should also be investigated for multiple species.

In regards to the behavior of cornea in compression, dynamic tests are needed in order to understand the changes in viscoelasticity as the sample loses water content during the compression process. In vivo the majority of compressive and some tensile and even shear forces are cyclic in nature. Examples of these are eye rubbing, and a temporarily increased intraocular pressure due to effects of the pulse. The effect of temperature variation on the elasticity as well as the effect of instant compression should also be investigated. Often used to investigate polymers, temperature sweep studies combine both the temperature behavior as well as the dynamic behavior [54, 55]. The effects on the temperature should also be investigated for the quasi-static type of experiments used in this thesis. The temperature effects if they exist will be very important to understand since the in-vivo and ex-vivo temperatures during tests may be quite different. Along with all the previously mentioned experiments, numerical models may also be of use with describing the similarities and differences between groups. They have the possibility of accomplishing this in two manners. Firstly, they will help to explain the behavior in terms of mathematical relationships which allows the physical behavior to be simplified and easier to manipulate. Secondly, with enough knowledge of how the corneal tissue behaves models may at least partially take the place of physical experiments. With further studies, a link between the more commonly studied tension behavior and compression

may be found. The effect of diameter within an unconfined compression test may also help to connect the results of compression experiments with more difficult inflation tests.

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